Considerations for the development of in vitro dissolution tests to reduce or replace preclinical oral absorption studies

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Abstract

The pharmaceutical development of new chemical entities can be hampered by their solubility and/or dissolution limitations. Currently, these properties are characterised mostly during in vivo pre-clinical studies. The development of appropriate in vitro methods to study the solubility and dissolution properties in preclinical species would lead to a significant reduction or replacement of the animal experiments at this stage of development. During clinical development, media simulating the human gastrointestinal tract fluids are commonly used and a similar approach mimicking laboratory animals gastrointestinal tract fluids would impact on the preclinical stage of development. This review summarises the current knowledge regarding the gastrointestinal physiology of the most common laboratory animals, and animal simulated gastric and intestinal media are proposed.

Keywords: animal, gastrointestinal physiology, biorelevant media, in vitro, dissolution testing

1. Introduction

When reviewing the properties of new chemical entities (NCEs) emerging from industrial drug discovery pipelines, many authors have commented on the increased number of molecules which possess challenging properties for drug development (Lennernas et al., 2014). Hydrophobicity and poor aqueous solubility are two properties which can compromise oral formulation development by impacting dissolution in the gastrointestinal tract and contribute to poor oral bioavailability (Stegemann et al., 2007). It is thus important to study these aspects early in the development process.

The solubility and dissolution rate limitations commonly found in NCE can be categorised by the Developability Classification System (DCS) (Butler and Dressman, 2010) which subcategorises class 2 compounds (low solubility, but high permeability) to class 2a (dissolution rate limited) or class 2b (solubility limited). Knowing the class and sub-class in which a compound resides can aid the decision on formulation strategy. Solubility and dissolution rate of a compound are often determined from in vitro solubility and dissolution tests conducted during physicochemical profiling (Markopoulos et al., 2015). Media selection is of critical importance when designing the in vitro test method. Since the late 1980's, in vitro methods have been developed with particular focus on using media that simulate human gastrointestinal fluid, known as biorelevant media, in order to improve in vitro-in vivo correlations (IVIVC), develop clinically relevant quality control methods and contribute to assessments of relative bioavailability and bioequivalence (Fotaki and Vertzoni, 2010; Gonzalez-Garcia et al., 2015; Wang et al., 2009). Over time, the complexity of these media has increased as more data have become available about gastrointestinal physiology. This is clearly illustrated by the sequential evolution of gastric and intestinal media, which have been developed by multiple groups to simulate the conditions of the human stomach and intestinal compartment in the fasted and fed states (Markopoulos et al., 2015).

Whilst the use of biorelevant media has improved the success rate of IVIVC and has contributed to formulation development strategies for clinical development projects, there remains a gap in terms of the application of a similar approach for pre-clinical formulation selection, particularly for oral toxicokinetic studies which are required to define exposure ranges for toxicology studies. The development of a pre-clinical in vitro dissolution test which could be used in combination with PBPK software to predict oral exposure at toxicologically relevant doses would facilitate a reduction in the number of preclinical in vivo studies which precede regulatory toxicology testing (McAllister, 2013). Such an approach would be in accordance with the 3Rs principle (Directive 2010/63/EU) which describes the need for the development of in vitro methods to substitute all or part of in vivo animal experimentation. Following the strategies for the development of biorelevant media to simulate gastrointestinal conditions in humans, the development of biorelevant media to simulate gastrointestinal conditions in animals could be a substantial contribution towards this goal.

In order to reduce or replace in vivo preclinical absorption studies with an in vitro test, the in vitro test method conditions in terms of media and hydrodynamics should be representative of the gastrointestinal environment of commonly used laboratory animals. Historically, in vitro test methods for studying dissolution performance, were devised for the purposes of quality control and regulation of pharmaceutical products, and not always for establishing the link to the pharmacokinetic parameters of the pharmaceutical product through its in vivo drug dissolution and release. As such these compendial methods do not adequately mimic the key processes involved in the in vivo absorption process in human or in animal, which is reflected by the poor correlations often found for poorly soluble compounds (Nicolaides et al., 2001). More recently, in vitro methods have been developed to address some of the key absorption differences, such as a dynamic pH environment or the absorption step with the use of

modified compendial or artificial gastrointestinal systems (Blanquet et al., 2004; Kostewicz et al., 2014; Kostewicz et al., 2004; Tsume et al., 2015).

In this review, we describe the physiological aspects of the gastrointestinal tract of dogs and rats, as the main two laboratory species used for preclinical oral absorption studies that should be considered for the development of biorelevant in vitro dissolution tests. The physiological data were reviewed alongside previously published compositions for animal biorelevant media and modifications/ new theoretical compositions are proposed to more accurately simulate the gastrointestinal fluids in both rat and dogs.

2. Gastrointestinal Anatomical and Physiological characteristics of Dogs and Rats relevant to drug dissolution

The impact of the physiological conditions of the gastrointestinal tract on dissolution and absorption of drugs has been discussed in detail in the literature. In summary, the pH of the fluid will influence the solubility of weak bases and acids. The presence of bile salts and phospholipids, through the formation of mixed micelles, will increase the solubility of poorly water-soluble drugs. Moreover, bile salts or phospholipids, decrease the surface tension of the medium (as amphiphilic molecules), which influences the dissolution of drugs (Fuchs and Dressman, 2014).

2.1 Stomach

After being chewed in the mouth and swallowed, a bolus of food is converted in the stomach to form chyme by the action of enzymatic digestion and stomach contractions from the three layers (longitudinal, circular and oblique) of smooth muscle of the stomach wall. Chyme is a

semi-liquid mixture of the gastric enzymes that are secreted in the stomach along with mucus, gastric acid, hormones and the ingested meal. The main function of the stomach is the production of chyme and its subsequent transportation into the small intestine where absorption of nutrients can take place. Minimal absorption of nutrients is found to occur from the stomach.

The anatomical structure of the dog stomach is regarded to be similar to the human stomach and it is a single compartment with a fluid capacity of 0.5-1L (McConnell et al., 2008; Sjogren et al., 2014).

The lumen of the rat stomach is different compared to the human stomach and it is composed of glandular and non-glandular compartments (de Zwart et al., 1999) that have a fluid capacity of 3.4mL (McConnell et al., 2008). Secretions in the rat stomach arise from the glandular portion, while the non-glandular part of the stomach is used for the storage and digestion of the food (Sjogren et al., 2014).

2.1.1 Gastric Volume: The fasted state gastric fluid volume that arises from ingestion and secretion is found to be between 10 and 50 mL in dog, 0.2 mL in rat, and below 50mL in human (McConnell et al., 2008; Rathbone and McDowell, 2013; Sjogren et al., 2014) (Table 1). In the fed state dog stomach, the total fluid volume is the sum of the ingested and secreted volumes. However, the secreted fluid volume is controlled by a neurohormonal response to the ingested meal and hence the total volume is quite variable. Because the rat is a continuous feeder, the secreted fluid volume is more consistent leading to total fluid volumes of about 1.3mL (McConnell et al., 2008). It is interesting to note that when reporting the ratio of the water volume of the stomach to body weight, a higher ratio is found in rat (3.2g/kg body weight) than in human (2.2g/kg body weight) (McConnell et al., 2008).

2.1.2 Gastric pH: High inter-subject variability for gastric fluid pH has been reported in human studies (Bergstrom et al., 2014). High variability of gastric pH was also found in laboratory animals (Arndt et al., 2013; Kararli, 1995; McConnell et al., 2008). In the fasted state dog, a broad span of values can be found in the literature, which range from pH 1.5 to 6.8. Several studies measured the pH of gastric aspirates and had reported relatively high values of pH 3 and above (Akimoto et al., 2000; Polentarutti et al., 2010; Vertzoni et al., 2007). However, a fasted pH value for dog of 1.5 is generally agreed, based on values obtained through pH telemetry capsule measurements (range 0.9-2.5) (Dressman, 1986; Lui et al., 1986; Mojaverian, 1996; Sagawa et al., 2009; Youngberg et al., 1985). When using pH telemetry capsules, the high variability observed in the dog could be due to the movements of the capsule inside the stomach (due to the migrating motor complex) (Sagawa et al., 2009; Sawamoto et al., 1997). The mean pH value in the fed state is 2.1 (Dressman, 1986). Unlike humans, there is no buffering effect of food measured in the dog's stomach after feeding, inducing less variation in the pH during postprandial phase. Moreover, the basal gastric secretion rate is lower in dogs than in humans (Dressman, 1986). The mean pH values in fasted and fed states for rats are 3.9 and 3.2 respectively (McConnell et al., 2008). In the study by McConnell et al., the authors postulate that the low content of protein in the animals' diet may explain a higher pH in fasted than fed state, by not stimulating a food buffering effect (McConnell et al., 2008).

2.1.3 Buffer Capacity: Very few data are available regarding the buffer capacity of the gastric fluids of laboratory animals. A study on fasted dogs showed a buffer capacity of 4 mmol/L/ΔpH (Vertzoni 2007), and 4.5 mmol/L/ΔpH in fed rats (Merchant 2015), which was

significantly different from the human median value (7-18mmol/L/ΔpH in fasted state, 14-28mmol/L/ΔpH in fed state) (Kalantzi et al., 2006a) (Table 1).

2.1.4 Osmolality: The osmolality of the fasted state stomach increases from dog to human to rat with values of 74.9mOsm/kg, 171-276mOsm/kg and 290mOsm/kg, respectively (Arndt et al., 2013; Mudie et al., 2010; Pedersen et al., 2013; Pihl et al., 2008). Similarly, the fed state gastric osmolality is higher in rat than in human (794 and 217-559mOsm/kg, respectively) (Merchant et al., 2015; Mudie et al., 2010). There are no data available for the osmolality values for the fed state in dog.

2.1.5 Surface tension: Similar values of surface tension are found for the fasted state in human and dog (41.9-45.7 and 33.3-43.3mN/m, respectively) (Mudie et al., 2010; Vertzoni et al., 2007). There are no data regarding the surface tension of the gastric fluids in the fasted state for the rat. In the fed state, surface tension values are close between human and rat (30-31 and 38mN/m, respectively), (Merchant et al., 2015; Mudie et al., 2010; Vertzoni et al., 2007). No data is available regarding the surface tension in fed state in dog.

2.1.6 Enzymes: The presence of enzymes in the stomach is essential for food digestion and can impact drug dissolution and stability. In dogs and in rats, pepsin and lipase are found in the stomach and their secretion and activity is increased in the fed state (Table 1). The same holds true for pepsin in humans (Mudie et al., 2010). Regarding gastric lipase in humans, the activity decreases 1h after meal intake, before increasing again reaching a value close to the fasted state (Armand et al., 1996).

2.1.7 Gastric motility and Gastric emptying rate: Dogs have a similar gastric motility pattern to humans with a fasted (preprandial) and fed (postprandial) state pattern. The fasted state motility consists of a two hour cycle, which comprises four phases (de Zwart et al., 1999; Dressman, 1986; Sjogren et al., 2014). Approximately half of the cycle duration is Phase 1, which is a quiescent phase where the stomach is mostly dormant and contractions are rare. During Phase 2, the frequency and intensity of the contractions gradually increase until reaching a maximum, which corresponds to Phase 3. This contractile activity of Phase 3 allows the stomach content to migrate to the small intestine through an interdigestive migrating motility complex (IMMC). An IMMC typically lasts for 20 minutes and spreads from the proximal stomach to the ileum every 1-2h (Sjogren et al., 2014). The transition from the strong contractile activity back to the quiescent phase is Phase 4 (de Zwart et al., 1999). During the fed state, the cyclic contractile motility pattern is replaced by regular tonic contractions. These contractions mechanically digest and mix the food with the gastric secretions to form chyme, which is then pushed towards the lower part of the stomach. Contractions of the lower part of the stomach allow the liquids and fine particles to pass into the duodenum, while larger particles are sent back to the body of the stomach. The motility pattern of the fasted state resumes when the meal is completely converted to chyme and has passed into the small intestine.

In both dog and human, the gastric emptying rate depends on the type of meal ingested (solid or liquid, nutrient or non-nutrient), but overall, the emptying rate is faster in dog than in human (Table 1). For non-nutrient liquids in dog, the emptying half-life is approximately four to five minutes, and for nutrient liquids values of twenty to twenty five minutes have been reported (Dressman, 1986). When compared to liquids, the emptying rate for solids in both

dog and human are considerably slower with an emptying half-life of ninety minutes for dog (Dressman, 1986).

Regarding gastric motility in rat, limited data are available, however, Sjogren et al reported a fasted state gastric emptying half-life of around 15 to 30 minutes for liquids (Sjogren et al., 2014). It should also be noted that rodents are continuous feeders unlike dogs or humans and hence a different motility pattern is expected.

2.2 Small intestine

The intestinal wall is composed of three layers: the mucosa, in contact with the chyme, the lamina propria, which contains mucosa-associated lymphoid tissue (MALT), and the muscularis, which has longitudinal and circular layers of smooth muscle. The mucosa contains several cell types, which exhibit different functions. These include goblet cells that produce mucus, endocrine cells that secrete hormones and peptides, immune cells (paneth cells) that produce protein rich material and protect the mucosa, and enterocytes (undifferentiated cells and absorptive cells) that allow the renewal of the mucosa and transport nutrients to the blood (de Zwart et al., 1999).

The small intestine is divided into three sections: the duodenum, the jejunum and the ileum. The length of the dog small intestine is strongly dependent on the breed, but is generally shorter than in human (3-5m in humans, 2.5-4.1m in dogs (Sjogren et al., 2014)) (Table 2). The rat small intestine is shorter than both human and dog with a typical length of 82cm (Clemens and Stevens, 1980).

2.2.1 Intestinal motility and Intestinal transit times: When considering transit time, the length of the intestine should be taken into account along with motility. In most species, the small intestine has two distinct motility patterns that are dependent on the prandial state. This intestinal motility mixes the chyme with bile salts and pancreatic enzymes and also moves this mixture down the digestive tract. In the fasted state, "housekeeping" contractions propagate from the stomach through the entire small intestine, pushing forward the intestinal contents (de Zwart et al., 1999). This motility typically results in a transit time through the dog small intestine of 2 hours, which is approximately half the transit time in human (Table 2) (Dressman, 1986). However, when considering the relative lengths of the dog and human small intestine, the transit rates are similar in the two species (de Zwart et al., 1999). In the rat, the intestinal transit time is similar to human (3 to 4 hours), hence the transit rate in rat is much slower in comparison to human or dog (Table 2).

In the fed state, the small intestine undergoes segmentation contractions and peristalsis. The segmentation contractions mix the chyme with the intestinal secretions and add mechanical sheer force to the digestion. Moreover, these contractions facilitate contact of the chyme with the gut epithelium, promoting the absorption process (de Zwart et al., 1999). The peristaltic contractions create pressure behind the volume of chyme enabling movement towards the anus (de Zwart et al., 1999).

2.2.2 Surface area: A further enhancement to the intestinal absorption process is the large surface area of the gut epithelium. The presence of numerous villi and microvilli significantly increases the surface of the intestine available for absorption ($54cm^2$/cm length jejunum, $38cm^2$/cm length ileum for dog, $1m^2$ absolute surface area for rat) (Hatton et al., 2015; Rathbone and McDowell, 2013).

2.2.3 Volume: The water volumes in the human small intestine were found to be 105 mL in the fasted state and a lower volume of 54 mL in the fed state (Schiller et al., 2005). Reported volumes in the dog small intestine were not found, but are expected to be equivalent to human. However, the equivalent volumes in rat were found to be higher in the fed state than the fasted state with reported values of 1.2 mL and 3.4 mL, respectively (McConnell et al., 2008). Similar to stomach volumes, the proportion of water volume in the intestine to body weight, was higher in rat than in human (11.1g/kg and 3.8g/kg respectively) (McConnell et al., 2008).

2.2.4 pH: Secretions from intestinal glands and from the pancreas increases the pH of the chyme coming from the stomach. This prevents irritation of the intestinal epithelium from elevated acidity levels, and produces optimal conditions for the enzymes.

A similar pattern of increase of pH values along the small intestine can be observed in humans and rats, both in fasted and fed state (human fasted: from 5.6 to 8.0, rat fasted: 5.89 to 5.93, human fed: 5.0 to 8.0, rat fed: 5.0 to 5.94) (Bergstrom et al., 2014; McConnell et al., 2008; Sjogren et al., 2014) (Table 2). The same is true for the dog in fasted state, with a pH increasing from 5.0 to 7.9 (Sutton, 2004) (Table 2). No values are available regarding the different pH values along the length of small intestine of the dog in fed state.

The intestinal pH is consistently 1 unit higher in dog than in human when comparing measurements at times normalized to gastric emptying of the pH measuring device. The duodenal pH in the fed state in dogs is lower than the duodenal pH in the fasted state and it decreases more rapidly and to a greater extent than in humans (change of pH from pH7 to

pH3 in 90min in dogs, compared to the pH change from pH6 to pH5 in 4h in humans) (Dressman, 1986).

2.2.5 Buffer capacity: The buffer capacity of the intestinal fluid in the dog is much lower than that of human, in the fasted state (Table 2). The buffer capacity was found to be 1.4mmol/L/ΔpH in dog, and values in human were found to vary from 3.2 to 6.4 mmol/L/ΔpH (Kalantzi et al., 2006a; Mudie et al., 2010). In the fed state, the buffer capacity is greater than in the fasted state, but decreases along the gastrointestinal tract in human (30 to 13.2 mmol/L/ΔpH) and rat (28.2 to 20.1 mmol/L/ΔpH) (Table 2) (Merchant et al., 2015; Mudie et al., 2010). However, the buffer capacity throughout the fed state dog small intestine is more constant with values of 24-30mmol/L/ΔpH (Kalantzi et al., 2006a).

2.2.6 Osmolality: Under fasted state conditions, the osmolality of the intestinal fluids in dog was reported to be ~70 mOsm/kg. In comparison, the osmolality found for the fasted state human intestine with duodenal fluids at 124-266 mOsm/kg, and a further rise to a value of 200-278mOsm/kg in the human jejunal fluids. The opposite was observed under fed state conditions, with values of 250-367 mOsm/kg in human duodenal fluid compared to the higher values of 667-841 mOsm/kg in dog intestinal fluid (Table 2) (Kalantzi et al., 2006a; Mudie et al., 2010). Osmolality values of the rat intestinal fluids in the fasted state were not found in the literature. The osmolality values of the rat intestinal fluids in the fed state were comparable to the osmolality values of the dog intestinal fluids and osmolality decreased from the proximal to the distal regions of the small intestine (896 to 546 mOsm/kg) (Merchant et al., 2015).

2.2.7 Surface tension: The reported values for the surface tension of the intestinal fluids were similar between human, dog and rat, both in the fasted and in the fed state (about 30mN/m) (Table 2) (Kalantzi et al., 2006a; Merchant et al., 2015; Mudie et al., 2010). An important element in the value of the surface tension is the presence of lipids. In dogs, the concentration of neutral lipids has been measured, in the fed intestine, at 12.2mM (Persson et al., 2005). In the fasted rat, the composition of fatty acids from the bile duct is: palmitic acid (31%), vaccenic acid (20%), linoleic acid (19%) and arachidonic acid (18.5%) (Ramaprasad et al., 2006).

2.2.8 Bile and phospholipids: An important element of intestine physiology, when considering drug dissolution is bile. By its properties of wetting and solubilisation, bile is a major factor in the digestion of fats and fat-soluble products. It is also involved in the elimination of many waste products into the bile and then in faeces (de Zwart et al., 1999). Bile is produced in the liver by the hepatocytes, and depending on the species, stored and concentrated in the gall bladder before being released in the intestine. Some anatomical differences have been noted between species, showing that dogs do not have a sphincter to regulate the release of bile into the intestine (de Zwart et al., 1999), and rats lack a gall bladder and present a diurnal rhythm (with highest flow at night) (Holm et al., 2013). In humans, bile is produced continuously (800mL/day), with a flow normalized to body weight of 1.5-15.4µL/min/kg (Holm et al., 2013). The bile flow is higher in rats (30-150µL/min/kg) than in dogs (13.2-25µL/min/kg) and humans (Holm et al., 2013; Rathbone and McDowell, 2013). Bile is a complex fluid containing water, electrolytes and organic molecules such as bile acids (water-soluble derivatives of cholesterol), cholesterol, phospholipids and bilirubin. Bile acids can be classified into two groups, primary and secondary bile acids. Primary bile acids are synthesized *de novo* from cholesterol in the liver *via* different pathways involving

many enzymes. Secondary bile acids are formed in the large intestine and the terminal ileum after bacterial hydrolysis, dehydroxylation, epimerisation and oxidation of hydroxyl groups (Holm et al., 2013). The secondary bile salts are absorbed and recirculated by the enterohepatic circulation. The main primary bile acids in mammalian species are cholic acid and chenodeoxycholic acid (Holm et al., 2013). In human, almost all primary bile acids (98%) are conjugated with amino acids in liver peroxisomes prior to their active secretion from the liver into the gallbladder and the small intestine (Holm et al., 2013). The hepatic bile salts are mainly conjugated by glycine, whereas in the duodenum, the bile acids are conjugated in the same proportions with glycine and taurine (de Zwart et al., 1999). In dogs, the bile salts are conjugated with taurine only (Falany et al., 1994), and the most abundant bile salt is taurocholic acid (Holm et al., 2013). The major bile acids in rats are taurine conjugated (Holm et al., 2013) with taurocholic acid as the main one (Sjogren et al., 2014). β-muricholic acid is also largely represented in the rodent bile (de Zwart et al., 1999). The differences in bile salt type and conjugation between dogs, rats and humans result in higher hydrophilicity values for dog and rat bile salts relative to their human counterparts (de Zwart et al., 1999; Holm et al., 2013) In the fasted state, the rat generates a higher bile salt concentration (17-61.3mM) than dog (2.4-10mM) or human (2.5-5.9mM in duodenum, 1.4-5.5mM in jejunum). In the fed state, higher concentration of bile salts are found in the dog intestine (8-18mM) than in human (3.6-24mM in duodenum, 4.5-8.0mM in jejunum) (Table 2) (Arndt et al., 2013; Bergstrom et al., 2014; Kalantzi et al., 2006a; Persson et al., 2005). In humans, dogs, as well as in rats the most common phospholipid in the bile is phosphatidylcholine, with a proportion of about 95% (Bergstrom et al., 2014), but the amount of phospholipids is higher in dog and rat than human (Bergstrom et al., 2014; Kalantzi et al., 2006a) (Table 2).

3. Biorelevant animal simulated gastrointestinal media

Human biorelevant media have been successfully applied to *in vitro* solubility and dissolution studies for improved bioprediction. Using a similar strategy for the development of biorelevant animal media, improved bioprediction could lead to a reduction in the use of animals in toxicology studies during the early stages of drug development.

As most drugs are developed for oral delivery, the focus for this study was to develop new simulated media for the stomach and small intestine fluids under fasted and fed state conditions, for both the dog and the rat. The development of the new media was based on existing published recipes. The main properties considered were: pH, osmolality, buffer capacity, surface tension, as well as composition and concentration of bile salts, phospholipids, fatty acids, ions, salts and enzymes.

Bile salts, phospholipids and fatty acids should be carefully selected to reflect the physiological components of gastrointestinal fluids and control surface tension. For example, lysophosphatidylcholine and the fatty acids; sodium oleate, glyceryl monooleate and palmitic acid are used to simulate the physiological enzyme degradation products (Arndt et al., 2013). With respect to bile salts, the use of pure bile salts is preferred to bile salt extracts in order to overcome issues of reproducibility related to variable composition between batches (Vertzoni et al., 2004). Concerning the type of bile salts, taurocholates are preferred as it has been noted that micelles from trihydroxy acids are relatively insensitive to changes in pH, ionic strength and temperature (Vertzoni et al., 2004). Bicarbonate salts are also found in the gastrointestinal tract, which are pH buffer components, but the technical difficulties related to their use has led to a preference for phosphate buffers in many simulated biological media (Sheng et al., 2009). The technical difficulties arise from a low stability of H_2CO_3, which decomposes at biological pH to form the poorly soluble gas CO_2. In order to retain the buffer

component HCO_3^- in the system CO_2 is sparged into the medium (Sheng et al., 2009), which causes a change to the pH. Therefore the stability of the pH is dependent on the rate at which CO_2 is sparged and is often found to be less stable than a phosphate buffer system. Furthermore, the subsequent formation of bubbles in the dissolution medium can cause mechanical stress and high variability in dissolution profiles (Boni et al., 2007). Even though commercially available setups make the use of bicarbonate buffers easier, it has been demonstrated that the use of non-physiologically relevant anions (such as phosphates) instead of bicarbonates in media will not impact on the dissolution of weak bases which have a pK_a below 5, but will influence the dissolution of highly lipophilic compounds with extremely low solubility (Vertzoni et al., 2004).

In this paper, we present published media recipes and discuss possible modifications. Further, we propose the theoretical composition of new media based on the available physiological data in order to simulate both the stomach and the small intestinal fluids of the dog and the rat in the fasted and fed state.

3.1 Canine fasted state simulated gastric fluid (cFaSSGF)

Modification of a medium already published in the literature is suggested based on the physiological values. A dog stomach simulated medium has been developed by Arndt and coworkers in 2013 (Arndt et al., 2013). A pH value of 1.5 (Table 3) reflects the strong acidity of the dog stomach (Dressman, 1986) and is prepared using concentrated hydrochloric acid (37%). Sodium chloride is used in the medium in order to achieve the desired osmolality.

Whilst bile salts, phospholipids and fatty acids are not produced in the stomach, they are often found to be present in a fasted gastric medium through a reflux mechanism from the

duodenum (Arndt et al., 2013). To represent bile reflux, a concentration of 0.2 mM of bile salt and 0.05 mM of phospholipid was added to the simulated gastric medium. These concentrations also maintain the 4:1 ratio of bile salts to phospholipids recorded in the canine intestinal fluid in the fasted state (Arndt et al., 2013).

It is proposed to modify the medium proposed by Arndt et al. to include the addition of enzymes in the medium. Based on the pepsin and lipase levels measured in the dog stomach (Table 1) in the studies of Magee and Naruse (1983) and Carriere et al., (1992), the addition of pepsin (600U/h,) and lipase (190U/h) to the medium is suggested (Table 3)(Carriere et al., 1992; Magee and Naruse, 1983). It is important to note that the optimal pH for pepsin activity is 2.0, and that gastric lipase is inactivated below pH 1.5 (Smeets-Peeters et al., 1998). Therefore pH should be carefully maintained in order to keep the enzymatic activity of the lipase, in the case that the enzyme is included in the medium when digestion of lipid based formulations is an important factor to be assessed.

3.2 Canine fed state simulated gastric fluid (cFeSSGF)

A medium simulating the dogs' stomach in the fed state has not been described in the literature. This medium would be highly dependent on the ingested meal. A medium representing the meal given to the animals or a milk-based medium could be used to simulate this physiological condition. The simulated media of the human gastric fluids in the fed state (FeSSGF) or long-life milk could be a good substitute for the dog's stomach fluids in the fed state (Dressman et al., 1998; Markopoulos et al., 2015). As the pH of these media is much higher than the pH of the dog's fed stomach the pH should be reduced at time 0, i.e. with the addition of an acidic solution of pepsin.

3.3 Canine fasted state simulated intestinal fluid (cFaSSIF)

A medium representing canine fasted state simulated intestinal fluid was published (Arndt et al., 2013). Here, taurocholic acid and taurodeoxycholic acid were found to be the most abundant tauro-conjugated bile acids at a concentration of 10mM in fasted state canine intestinal fluid (Arndt et al., 2013; Falany et al., 1994; Holm et al., 2013). Hence, the bile salt sodium taurocholate and sodium taurodeoxycholate were prepared to a concentration of 5mM each (10mM total) (Table 3). Based on this bile salt concentration, and the 4:1 ratio between bile salts and phospholipids, the phospholipid concentration of the medium was 2.5mM, using equimolar concentrations of phosphatidylcholine and lysophosphatidylcholine (Table 3). Sodium oleate was included as a product of lipolytic activity, in equimolar concentration to lysophosphatidylcholine (Arndt et al., 2013). The combination of the two bile salts with the use of lysophosphatidylcholine and sodium oleate results in the desired surface tension of 41.9-45.7 mN/m measured in the dogs intestinal fluids in the fasted state (Arndt et al., 2013). Sodium phosphate buffer, sodium hydroxide and sodium chloride were used to control the desired osmolality and buffer capacity at pH 7.5.

In order to take into account the variability and reflect the distribution of dog intestinal pH values reported in the literature a modification from pH 7.5 to pH 6.8 (median pH value in fasted intestinal canine lumen) is proposed (Table 2), with a possible impact on solubility of weak acids. The values for all the other components and properties of the medium published by Arndt et al. (2013) are physiologically relevant.

3.4 Canine fed state simulated intestinal fluid (cFeSSIF)

A medium simulating the dog intestine in the fed state has not been described in the literature and a novel medium is proposed based on reported values of the characteristics of the dog

intestinal contents in the fed state. The pH of this medium is set at 6.3, as this was the median value reported in the literature, and a phosphate buffer or maleate buffer is suggested (Diem, 1962). In an article by Persson and coworkers, an extract of the dog intestinal contents in the fed state was found to have a bile salt concentration of 5 mM. As in the dog intestinal fluids in the fasted state, the main two bile acids were found to be taurocholic acid (74%) and taurodeoxycholic acid (21%) (Persson et al., 2005). But, the relative percentage had changed from 50% of both bile salts in the fasted state to 74% sodium taurocholate and 21% sodium taurodeoxycholate in the fed state. For simplicity, 75% and 25% were used, leading to concentrations of 3.75 and 1.25 mM, respectively to account for the total 5mM bile salt concentration found. A bile salt:phospholipid ratio of 4:1 was reported, indicating a 1.25 mM phospholipid concentration in the dog intestinal fluids in the fed state, with the lysophosphatidylcholine and phosphatidylcholine being the main ones (Persson et al., 2005). However, a lower bile salt:phospholipid ratio of 1:1 was reported by Kalantzi et al (Kalantzi et al., 2006b). Hence, an average bile salt:phospholipid ratio of 2.5:1 was selected (5mM:2mM). Fatty acids at a concentration of 12.2 mM were measured in the dog intestinal fluid in the fed state (Persson et al., 2005). As a suitable fatty acid was not specified in the literature for the dog intestinal fluid glyceryl monooleate was selected, as this fatty acid has been suggested in the fed state human simulated intestinal fluid, (FeSSIF-V2, (Jantratid et al., 2008) (Table 3).

3.5 Rat fasted state simulated gastric fluid (rFaSSGF)

A medium simulating the rat stomach in the fasted state has not been described in the literature and a novel medium is proposed based on reported values of the characteristics of

the rat gastric contents in the fasted state. The pH is set at 3.9, which was based on the physiological value determined by McConnell et al (McConnell et al., 2008) (Table 4).

Bile reflux is known to occur in the rat and hence a bile salt concentration of 4mM is suggested based on the physiological values (Tanaka et al., 2014). Sodium taurodeoxycholate was not found to be in significant quantities in the rat bile duct, therefore only sodium taurocholate was selected (Alvaro et al., 1986). The bile salt:phospholipid ratio was found to be significantly greater in rat than in dog with reported values of 23:1 (Tanaka et al., 2012). As such a 0.2 mM concentration of phospholipid is proposed. Only trace quantities of lysophosphatidylcholine were detected in rat gastric fluid, therefore only phosphatidylcholine is the proposed phospholipid for this medium (Alvaro et al., 1986). Regarding enzymes, in fasted state, the secretion of pepsin in the rat stomach is 1.2µg/h (Shahroki et al., 2015), (Table 4). Lipase activity has been measured at 44.3U/h (Levy et al., 1981) (Table 4).

3.6 Rat fed state simulated gastric fluid (rFeSSGF)

A medium simulating the rat stomach in the fed state has not been described in the literature. A medium representing the meal given to the animals, or a buffer of pH 3.2 (reflecting the physiological pH value of the rat stomach) with the addition of sodium taurocholate, phosphatidylcholine and fatty acids, which have been identified (but not quantified) in the rat stomach fluids in the fed state, are suggested. To the best of our knowledge, no data are available regarding the concentrations of these components in the rat stomach fluids in the fed state.

3.7 Rat fasted state simulated intestinal fluid (rFaSSIF)

Modification of a medium already published in the literature is suggested based on the physiological values. A medium to simulate the rats' intestinal fluid in the fasted state has been proposed by Tanaka et al. (2014). The first modification proposed refers to the pH of the medium. Based on the median value of the pH of the rats intestinal fluids in the fasted state (Table 2) a modification from pH 7.0 (value based on measurements at 10-15 min intervals over 75 min after administration of 1mL ultrapure water) (Tanaka et al., 2014) to pH 6.0 is proposed. 0.2 M sodium dihydrogen phosphate, 0.2 M acetic acid and 0.2 M sodium hydroxide are suggested as the buffer system for the desired pH value (pH 6.0). A concentration of 50mM of sodium taurocholate is used in the published medium, that is based on the measured concentration of bile acids in the upper jejunum (Tanaka et al., 2012) and taurocholic acid was found to be the main bile acid in the rat intestine (Sjogren et al., 2014). The second modification proposed refers to the phospholipid concentration in the medium. In the published medium a 3.7mM egg phosphatidylcholine is suggested as the phospholipid for the medium. Based on the physiological value for the bile salt:phospholipid concentration ratio of 23:1 we suggest the addition of 2.2 mM phosphatidylcholine in the medium (Tanaka et al., 2012) (Table 4).

3.8 Rat fed state simulated intestinal fluid (rFeSSIF)

A medium simulating the rat intestine in the fed state has not been described in the literature and a novel medium is proposed based on reported values of the characteristics of the rat intestinal contents in the fed state. The pH is set at 5.5, as the physiological pH values range from a value of 5.0 for the duodenum to a value of 5.94 for the ileum, as stated in McConnell et al.(McConnell et al., 2008) (Table 2). Sodium taurocholate and phosphatidylcholine are suggested in order to represent the main bile salts and phospholipids and are set at

concentrations of 13.7 mM and 6.3 mM, respectively (Table 4). With respect to fatty acids, palmitic acid is proposed as the representative fatty acid as it was found to be the main component (31%) measured in the bile duct of fed rats (Ramaprasad et al., 2006) [in terms of simplification of the medium the addition of one fatty acid is proposed]. As there are no information available regarding the fatty acids' concentration, a concentration of 18.3mM is proposed, based on the monoglycerides-fatty acids/phospholipids ratio (2.9:1) that is used in the human fed state simulated intestinal fluid (FeSSIF-V2) (Jantratid et al., 2008).

4. Conclusions

In the last decades, several media simulating the human gastrointestinal tract have been developed and successfully used. However, limited information is available for media to simulate the gastrointestinal tract of laboratory animals. This review summarises the limited available media mimicking dogs and rats digestive tract, suggesting modifications, and proposes novel ones based on the most recent physiological data available. The use of these media would support the 3Rs as well as it would be used as a tool to develop in vitro in vivo correlations. Further studies which will assess the potential of using these newly developed media with a novel mini-scale dissolution method to improve the prediction of oral formulation performance in preclinical species are in progress.

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Tables

Table 1: Comparative Anatomical and Physiological characteristics of the Stomach in humans, dogs and rats

		Human	Dog	Rat
Gastric emptying time		t1/2 liquids: 8-15 min a t1/2 meal: 30min-3h b	t1/2 liquids: 4-5 min a t1/2 meal: 90min a	t1/2 meal: 15–30 min b
Water volume		<50mL (fasted) b Up to 1L (fed) b	Similar to humans especially for dogs >20kg b	0.2mL (fasted) c 1.3mL (fed) c
pH	Fasted	1.7-3.3 d	1.5±0.04 a	3.9 c
	Fed	3.5 e	2.1 a	3.2 c
Osmolality (mOsm/kg)	Fasted	171-276 f,g	74.9±6.0 h	290 i
	Fed	217-559 f		794±260 j
Surface tension	Fasted	41.9-45.7 f	37.3 (33.3-43.3) k	
	Fed	30-31 f		38±2 j
Buffer capacity (mmol/L/ΔpH)	Fasted	7-18 l	4.0 (0.6-6.6) k	
	Fed	14-28 l		4.5±1.9 j
Enzymes	Pepsin	81mg/h v, 0.1-1.3mg/mL (fasted) f, 273-339 mg/h v, 0.26-1.72 mg/mL	600U/h (fasted) q 1.56±0.60mg/h for the first hour and 0.56±0.15mg/h for the second hour	12µg/mL, 1.2µg/h (fasted) s, u

		(fed) f	(fed) r	
	Lipase	≈ 43.9 U/mL o, 0.1mg/mL (fasted) m 11.4-43.9U/mL (fed) o	190U/h (basal secretion) n, 7.2mg over 3h digestion (fed) p	44.3U/h/g wet tissue t

729 a)(Dressman, 1986), b)(Sjogren et al., 2014), c)(McConnell et al., 2008),
730 d)(Bergstrom et al., 2014), e)(de Zwart et al., 1999), f)(Mudie et al., 2010),
731 g)(Pedersen et al., 2013), h)(Arndt et al., 2013), i)(Pihl et al., 2008), j)(Merchant et
732 al., 2015), k)(Vertzoni et al., 2007), l)(Kalantzi et al., 2006a), m) (Carriere et al.,
733 2000), n)(Carriere et al., 1992), o) (Armand et al., 1996), p) (Carriere et al., 1993),
734 q)(Magee and Naruse, 1983), r) (Kondo et al., 1994), s)(Asokkumar et al., 2014),
735 t)(Levy et al., 1981), u) (Shahroki et al., 2015), v) (Lentner, 1981)

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739 **Table 2: Comparative Anatomical and Physiological characteristics of the Small**

740 **Intestine in humans, dogs and rats**

		Human	Dog	Rat
Length		3-5m a	2.5-4.1m a	0.82m b
Absorbing surface		200m^2 c	54cm^2/cm length jejunum, 38cm^2/cm length ileum d	1m^2 e
Small intestine transit time		4h (fasted or light meal) f	2h (fasted) f	3-4h (fasted) a
Water volume	Fasted	105mL g		1.2mg h
	Fed	54mL g		3.4mL h
pH	Fasted	5.6-7.0 (duodenum); 6.0-7.8 (jejunum); 6.5-8.0 (ileum) a, i	5.0-7.6 (duodenum), 6.2-7.3 (jejunum), 6.6-7.9 (ileum) j	5.89 (duodenum), 6.13 (jejunum), 5.93 (ileum) h
	Fed	5.0-6.5 (duodenum); 5.0-6.5 (jejunum); similar to fasted (ileum) a, i	5.0 (duodenum) j	5.0 (duodenum), 5.10 (jejunum), 5.94 (ileum) h
Osmolality (mOsm/kg)	Fasted	124-266 (duodenum), 200-278 (jejunum) k	~70 l	
	Fed	250-367 (duodenum)	667-841 l	896±104

		k		(proximal), 640±73 (mild), 546±62 (distal) m
Buffer capacity (mmol/L/ΔpH)	Fasted	5.6 (duodenum), 3.2 (jejunum), 6.4 (ileum) k	~1.4 l	
	Fed	18-30 (duodenum), 13.2-14.6 (jejunum) k	24-30 l	28.2±0.8 (proximal), 22.7±2.4 (mild), 20.1±0.7 (distal) m
Surface tension (mN/m)	Fasted	33.3-46.0 (duodenum), 28 (jejunum) k	~31 l	
	Fed	32.2-36.7 (duodenum), 27 (jejunum) k	~28 l	33±1 (proximal), 35±1 (mild), 39±5 (distal) m
Bile salts	Fasted	2.5-5.9mM (duodenum), 1.4-5.5mM (jejununm) i	2.4-10mM l, n	17-61.3mM i
	Fed	3.6-24.0mM (duodenum), 4.5-8.0mM (jejunum) i	8-18mM l, o	12.2-15.1mM p

Phospholipids	Fasted	0.26mM (duodenum), 0.19mM (Jejunum) i	Low l	6.2-6.5mM i
	Fed	1.2-6.0mM (duodenum), 2.0-3.0mM (jejunum) i	4.36-19.4mM l	

a) (Sjogren et al., 2014), b) (Clemens and Stevens, 1980), c) (DeSesso and Jacobson, 2001), d) (Rathbone and McDowell, 2013), e) (Hatton et al., 2015), f) (Dressman, 1986), g) (Schiller et al., 2005), h) (McConnell et al., 2008), i) (Bergstrom et al., 2014), j) (Sutton, 2004), k) (Mudie et al., 2010), l) (Kalantzi et al., 2006b), m) (Merchant et al., 2015), n) (Arndt et al., 2013), o) (Persson et al., 2005), p) (Hagio et al., 2009)

749 **Table 3: Composition and Physicochemical properties of the Canine Simulated Media**

	cFaSSGF	cFaSSIF	cFeSSIF
pH	1.5	6.8	~6.3
Bile salts	Sodium taurodeoxycholate (0.1mM), sodium taurocholate (0.1mM)	Sodium taurocholate (5.00mM), sodium taurodeoxycholate (5.00mM)	5mM (3.75mM taurocholic acid; 1.25mM taurodeoxycholic acid)
Phospholipids	Phosphatidylcholine (0.025mM), lysophosphatidylcholine (0.025mM)	Phosphatidylcholine (1.25mM), lysophosphatidylcholine (1.25mM)	2mM phosphatidylcholine
Fatty acids	Sodium oleate (0.025mM)	Sodium oleate (1.25mM)	12mM Glyceryl monooleate
Buffer, Cations, salts	Hydrochloric acid, sodium chloride (14.5mM)	Sodium dihydrogen phosphate monohydrate (28.65mM), sodium hydroxide (28mM), sodium chloride (59.63mM)	0.07M monopotassium phosphate;0.07M disodium phosphate or 0.2M Tris acid maleate; 0.2N NaOH
Enzymes	pepsin (600U/h), lipase (190U/h)		

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752 **Table 4: Composition and Physicochemical properties of the Rat Simulated Media**

	rFaSSGF	rFaSSIF	rFeSSIF
pH	3.9	6.0	5.0-5.5
Bile salts	4mM taurocholic acid	50mM Taurocholic acid	13.7mM taurocholic acid
Phospholipids	0.2mM phosphatidylcholine	2.2mM phosphatidylcholine	6.3mM phosphatidylcholine
Fatty acids			18.3mM palmitic acid
Buffer, Cations, salts	0.1M acetic acid; 0.1M sodium dihydrogen phosphate	0.02M acetic acid; 0.02M sodium dihydrogen phosphate, 0.02M sodium hydroxide	
Enzymes	Pepsin (1.2µg/h), lipase (activity 44.3U/h)		

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